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Surfaces in seawater are quickly covered by complex organic films which greatly influence subsequent microbial colonization. We have focussed on one predominant component of the organic film, proteins. During this first year, we found that the mechanism of protein adsorption in seawater is similar to that observed in non-marine systems, but unlike non-marine systems, protein desorption can be substantial at high protein concentrations. We also found that the micron-scale distribution of adsorbed proteins varied with different surfaces. Finally, using an immunological assay, we measured dissolved concentrations of ribulose-bisphosphate carboxylase (RuBPCase), which is the most abundant protein in nature. RuBPCase comprises about 2% of the total dissolved protein pool, a high percentage considering the large number (1×10^6) of possible proteins in seawater. In addition, immunological approaches are being used to examine protein adsorption and degradation in natural microbial films.

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Yearly Progress Report

Contract N00014-87-K-0108 R&T Code 441h001

Principal Investigators: David Kirchman and Stephen Dexter

Contractor: University of Delaware

Contract Title: Protein Adsorption and its Role in Bacterial Film Development

RESEARCH OBJECTIVES: To investigate the mechanisms of protein adsorption, the effect of adsorption on protein conformation, and relationships among surface properties, protein conformation, and bacterial film development on surfaces placed in seawater.

PROGRESS: During this first year of our project, we have made progress in three areas of the originally proposed research; 1) mechanisms of protein adsorption in seawater; 2) the effect of adsorbed proteins on bacterial film development; and 3) the use of immunological techniques to examine dissolved and adsorbed proteins and their degradation by bacteria in seawater.

I. Protein Adsorption in Seawater

Because of the probable importance of proteins in bacterial biofouling and other microbial processes, we examined some of the possible mechanisms for protein adsorption in seawater. There is an extensive literature about protein adsorption in non-marine systems, yet it was not immediately clear that all of these findings could be applied to adsorption in seawater. The most important mechanism for protein adsorption that has been examined extensively in non-marine application involves hydrophobic interactions. We found that these interactions were even more important in seawater. These results have been described in a paper that is near submission (Kirchman et al. in prep.).

We examined two proteins, bovine serum albumin (BSA) and the CO_2-fixing enzyme, ribulose-bisphosphate carboxylase (RuBPCase). RuBPCase is one of the most abundant proteins in nature, but its adsorption properties have not been studied previously. BSA was chosen because of the large literature on this protein. These two proteins were ^{3}H-labelled by reductive methylation. Two types of experiments indicated that adsorption of ^{3}H-proteins was similar to that of unlabelled proteins.

Adsorption Rate and Concentration Dependence Protein adsorption in seawater is very rapid. About 50% of maximum adsorption is observed within seconds of immersing surfaces in protein solutions. Adsorption increased with protein concentration in the bulk solution. However, adsorption at high concentrations is reversible, unlike that observed in low ionic strength media. When protein solutions were replaced with protein-free seawater, adsorbed proteins released rapidly into solution. At high initial concentrations (5 mg/ml), 80% was

desorbed within 1 min. At low initial concentrations (0.01 mg/ml), however, desorption was minimal (<20%). In all subsequent experiments, we used low protein concentrations to avoid complications caused by desorption and to mimic more closely conditions actually observed in nature.

Hydrophobicity vs. Ionic Effects We measured adsorption of RuBPCase to a variety of surfaces which differed in composition and surface energies. Work of adhesion was estimated from measured water contact angles. A short paper on characterization of the surfaces is being prepared by Dexter and McDonald. RuBPCase adsorption in seawater was significantly higher for hydrophobic surfaces with low work of adhesion. In contrast, RuBPCase adsorption in low ionic strength buffer (pH 8.2, same as seawater) onto the various surfaces did not differ significantly. These results point to the importance of hydrophobic interactions in governing protein adsorption in seawater.

We consistently observed that adsorption of RuBPCase and BSA to any surface is higher in seawater compared with low ionic strength buffer. The difference was entirely due to the higher ionic strength of seawater. Adsorption increases with concentrations of either NaCl or $MgCl_2$. Especially in low salt concentrations, adsorption was higher in $MgCl_2$ than in NaCl.

The lack of a relationship between surface energy and protein adsorption in buffer and the enhancement of adsorption with low concentrations of $MgCl_2$ indicate the importance of ionic interactions when the ionic strength of the bulk solution is low. In this case, the addition of divalent cations like Mg^{+2} allows the Guoy-Chapman double layer to form, which facilitates adsorption via ionic interactions. However, at high ionic strengths these interactions are reduced. Rather, protein adsorption is high because of the "salting out" of proteins, i.e., increased protein-protein and protein-surface interactions at the expense of protein-solvent interactions.

Specificity of Protein Adsorption We observed some differences between BSA and RuBPCase adsorption, suggesting that the binding mechanisms for these proteins may differ. To examine in further detail the generality of adsorption mechanisms, we tested whether or not the different proteins bind to the same sites on glass and parafilm. We pre-exposed these surfaces to a variety of proteins, rinsed, and then exposed the surfaces to ^{3}H-BSA. We found that all proteins tested (including RuBPCase) inhibited BSA adsorption to glass and parafilm. Also, pre-exposure of surfaces to glycine had little effect. These results suggest that all proteins bind to the same sites on surfaces but to different sites than those of free amino acids, assuming glycine binds at all.

II. Relationship between Adsorbed Proteins and Bacterial Colonization of Surfaces in Seawater

Previous work indicated that bacterial colonization could be predicted from neither surface properties (e.g. surface energy) nor the presence of adsorbed organic matter alone. One of our general goals was to explore how the conformation of the adsorbed organic matter, in our case proteins, is changed by the surface and to determine how, if at all, these changes affect bacterial colonization and growth on surfaces. Our definition of "conformation" is

broader than that used by protein biochemists. In addition to the molecular arrangement of proteins on surfaces, we are interested in the micron-scale distribution of adsorbed proteins and the availability of these proteins to proteases and bacterial degradative enzymes. We examined some of these issues during the past year.

Micron-scale Distribution of Adsorbed Proteins For our experiments examining protein adsorption onto different surfaces, we measured total adsorption onto four 1 X 1 cm squares and calculated an average adsorption per cm^{-2}. However, it is possible that proteins do not adsorb evenly over the entire 1 X 1 cm square, and furthermore that the micron-scale distribution of proteins may differ on various surfaces even if the average adsorption is similar. Given that natural marine bacteria are on the order of 1 μm, the micron-scale distribution is likely to be more relevant to how adsorbed proteins affect bacterial attachment than the average protein amount adsorbed per cm^{-2}.

In order to examine the micron-scale distribution, surfaces were exposed to BSA, rinsed, and then stained with the fluorochrome, fluorescein isothiocyanate (FITC), which binds specifically to proteins. The stained surfaces were then examined under epifluorescence microscopy. The images were analyzed with an Olympus Cue image analysis system to determine number and size of the fluorescing protein patches.

BSA added to seawater does not bind evenly, nor is the pattern of adsorption similar on the different surfaces we examined. Rather, adsorption was patchy, and the size and distribution of protein patches varied with different surfaces. On polystrene and polyvinyl fluoride (PVF), the patches were evenly distributed with an average size of about 10 μm^2. In contrast, on teflon (PTFE), the patches varied greatly in size and were unevenly distributed on the surface. Patch size varied from $<1\ \mu m^2$ to $>100\ \mu m^2$.

We don't know the causes for the heterogeneity of protein adsorption on all the surfaces examined to date. Although we are not totally sure about the differences among the three surfaces examined, the highly irregular distribution of adsorbed proteins on teflon may be due to the very low critical surface tension of this material; it is a hydrophobic surface. We are currently exploring the consequence of these results for understanding bacterial colonization. Specifically, we are examining whether bacteria attach to protein patches or to bare surfaces between protein patches.

Availability of Adsorbed Proteins Previous studies on protein adsorption in non-marine systems found that the amount of adsorbed protein that could be removed by detergents varied with different surfaces. These results suggested that availability of adsorbed proteins for bacterial use may differ among the various surfaces. In fact, we found that RuBPCase adsorbed to glass was nearly completely hydrolyzed by the protease trypsin, while this protease was only 50% effective in hydrolyzing adsorbed RuBPCase from parafilm, which is a hydrophobic surface. The opposite result was observed when RuBPCase-coated surfaces were exposed to seawater containing natural bacterial assemblages. Bacteria were able to degrade nearly all of the adsorbed protein from the hydrophobic surface, but were only 50% effective in removing adsorbed protein from the hydrophilic surface.

Clearly, bacteria differed from proteases in their effectiveness in degrading adsorbed proteins because bacteria are more than just proteases. What is not so clear is why bacteria were not as able to degrade protein adsorbed to hydrophilic surfaces as from hydrophobic surfaces. Part of the answer may come from our experiments examining the effect of adsorbed proteins on bacterial colonization. We found that adsorbed protein can inhibit bacterial colonization to glass, a hydrophilic surface. The mechanisms behind these observations are still obscure and are being investigated.

III. Immunological Examination of Proteins In Seawater

Little is known about proteins that are dissolved or adsorbed to surfaces in seawater. Chemical oceanographers measure the concentration of dissolved combined amino acids, which includes polypeptides, but also free amino acids adsorbed to particle surfaces. To begin to characterize proteins in seawater and to develop techniques for examining adsorbed proteins, we have raised antibodies to two abundant proteins in phytoplankton, the most likely source of proteins in the sea. These two proteins are RuBPCase (used in adsorption studies mentioned above) and a pigment-binding protein (LHC) which is involved in light harvesting for photosynthesis. LHC is an abundant complex of three membrane polypeptides proteins (ca. 20 kD) which we think will offer interesting contrasts with RuBPCase, a soluble protein. Antibodies are only now just available for LHC, so most of our work has been with RuBPCase.

Our first step was simply to determine whether or not RuBPCase was present dissolved in seawater. Samples (5 liters) were concentrated by ultrafiltration and total protein concentrations were measured. The concentration step is about 85% efficient in collecting the >10 kD fraction (particles >0.2 µm were removed by filtration). Subsamples of the >10 kD fraction were subjected to a dot-blot assay using an enzyme-linked detection system. We found that RuBPCase concentrations vary seasonally and are about 1 µg/liter, which is about 2% of the total protein concentration found in the Delaware Bay. This percentage is actually large considering the large number of possible proteins found in seawater (>1.0 X 10^6).

Currently, we are using these antibodies to examine the spatial distribution of RuBPCase among other proteins adsorbed from natural seawater samples. We are also examining one possible source of proteins, fecal pellets produced by copepods grazing on algae. Immunological techniques, such as those applied in our studies, appear to be a powerful approach to examine specific proteins in complex mixtures.

Publications and Reports

Kirchman, D., D. Henry, and S. Dexter. Protein adsorption in seawater. Manuscript exists, to be submitted by July 15.

Kirchman, D. "Mechanism of Protein Adsorption in Seawater and Effect of Adsorbed Proteins on Bacterial Attachment", ONR Workshop on Fouling, University of Southern California, November 1987.

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